ORIGINAL ARTICLE

RECORDS OF PHARMACEUTICAL AND BIOMEDICAL SCIENCES

Detection of Recombinant Erythropoietin Biosimilars and Analog Substances in Human Urine by Gel Electrophoresis Technique

Ahmed A. Abdallah[1*], Alaa S. Wahba[2], Asmaa R. Abdel-Hamed[2], Noha M. Mesbah[2], Dina M. Abo-elmatty[2]

[1] Medical Supply Department, International Medical Center, Kilo 42, Cairo-Ismailia Desert Road, Egypt;
[2] Department of Biochemistry, Faculty of Pharmacy, Suez Canal University, Ismailia, 41522, Egypt

Received: 22. 9. 2024

Revised: 9. 10. 2024

Accepted: 14. 10. 2024

*Correspondence Author:

Tel: +201273224663

E-mail address:

ahmed.anas24@gmail.com

Abstract

This study aimed to improve the detection of erythropoietin (EPO) and its biosimilars in human urine using the SAR-PAGE technique by electrophoresis method in pre-casted gels to help differentiate each biosimilar of EPO according to their molecular masses along with their electrophoretic characters. 20 ml human urine samples were collected, purified then spiked with the known concentration of erythropoietin standards. SAR-PAGE electrophoresis was applied using two different concentrations of N-Lauroylsarcosine sodium salt to visualize the difference and effect of sarkosyl on human erythropoietin detection, followed by immunodetection using chemiluminescence reagent. All data were collected, tabled, and statistically analyzed. The first group (using 0.1% w/v SARCOSYL-PAGE) showed large condensed shadowed bands and asymmetric bands behavior while the second group (using 0.12 % w/v SARCOSYL-PAGE) showed confirmed, thin,sharp bands and symmetric identical bands behavior. Increasing sarcosyl concentration to 0.12 % w/v affects the immunological detection by increasing the affinity of the monoclonal anti-EPO antibody to the protein chain in both PEGylated and non-PEGylated epoetins thus leading to enhanced antibody binding and a sharper electrophoretic separation and detectable bands.

Keywords: Doping; Mircera; SARCOSYL-PAGE; Immunopurification; Chemiluminescence.

1. Introduction

Erythropoietin, also known as erythropoietin, haematopoietin, or haemopoietin, is a glycoprotein cytokine secreted mainly by the kidneys in response to cellular hypoxia. It stimulates the bone marrow to produce red blood cells (erythropoiesis) **(Zivot et al., 2018)**. Erythropoietin is produced by interstitial fibroblasts in the kidney in close association with the peritubular capillary and proximal convoluted tubule. It is also produced in perisinusoidal cells in the liver. Liver production predominates in the fetal and perinatal periods, while renal production predominates in adulthood. It is homologous with thrombopoietin **(Jelkmann, 2013)**.

Since been first approval in 1989, and rather than its misuse for doping purposes, recombinant erythropoietin has been used mainly for the stimulation of erythropoiesis in anemia caused, for example, by chronic kidney disease or chemotherapy. The detection of recombinant

peptide and protein hormones in doping – for example erythropoietin (EPO) and human growth hormone (hGH) – is generally one of the most challenging analytical problems in doping control. The World Anti-doping Agency's (WADA) accredited method for the detection of doping with recombinant human erythropoietin (rhEPO) is based on isoelectric focusing (IEF) **(Reihlen et al., 2012)**. In addition, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has proven a valuable tool for detecting Dynepo doping with increased sensitivity and is also useful for ruling out active and effort-type urines **(Kielkopf et al., 2021)**.

However, both the SDS-PAGE and the IEF-PAGE methods use urine as a sample matrix for the detection of EPO and its biosimilars. Unfortunately, continuous erythropoietin receptor activator (CERA), one of the latest generations of EPO-based pharmaceuticals, which is a PEGylated protein, marketed under the brand name MIRCERA, a-PEGylated protein beta) is difficult to be excreted in urine because of its extended serum half-life (approximately 130 h) and molecular weight (approximately 60 kDa). In consequence, up to four methods have to be performed to detect clearly and confirm the misuse of recombinant erythropoietin **(Desharnais et al., 2013)**. The SDS-PAGE method is the same as ELISA or IEF methods detecting erythropoietin in urine but unfortunately, SDS-PAGE method turned out to be less sensitive for MIRCERA than for the other rhEPO **(Cavalcanti et al., 2019)**.

In the present work, the reason for this altered behavior was identified and a new electrophoretic method with specifically enhanced sensitivity for MIRCERA was developed without altering the performance characteristics of SDS-PAGE for other epoetins by using the SAR-PAGE technique.

2. Materials and Methods

2.1. Materials

EPO_MAIIA Purification kit was purchased from MAIIA AB, Uppsala, Sweden. Sample Buffer for SAR-PAGE: N-Lauroylsarcosine sodium salt, serum bovine albumin solution (BSA) 0.1%, phosphate buffered saline (PBS) 1x, methanol for liquid chromatography, DTT: DL-Dithiothreitol, sodium metabisulfite, 7% & 0.7% acetic acid solution, Trisma HCL, Trisma base, MOPS, EDTA, glycerol, glycine and phenol red were supplied by Sigma-Aldrich Chemie GmbH. molecular weight marker: protein marker VI (10 - 245) prestained was purchased from AppliChem GmbH. Primary Antibody: anti-human EPO monoclonal mouse IgG CLONE AE7A5 was purchased from R&D Systems, Biotechne, USA. Secondary antibody: goat anti-mouse + HRP conjugate was supplied by Merck, DARMSTADT. Chemiluminescence reagent solution: supersignal® west femto maximum sensitivity substrate was purchased from Thermo Fisher Scientific.

2.2. Solutions and control samples

Eprex 25 pg/ µL alpha and beta rEPO, 40 pg / µL NESP (AranespTM), CERA (MIRCERATM) of 333 ng / µL were purchased from local Egyptian pharmacy store. EPO-Fc of 20 pg / µL was purchased from cell sciences online store.

2.3. Samples collection

Urine samples were collected from 20 healthy male volunteers aged 25-30 years and distributed into aliquots of 20 ml at the same time +ve control samples were prepared freshly at room temperature. All experimental procedures were approved by the research ethics committee at Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt (202106MH2).

Samples were distributed into 2 groups (one group with 0.1% w/v sarcosyl and second group with 0.12% w/v sarcosyl).

2.4. Immunopurification in MAIIA columns

The procedure was carried out as described in the instructions in the MAIIA EPO Purification Kit (Art. No. 1390) (MAIIA Diagnostics, Uppsala Sweden). Samples were filtered through a filter with PVDF membrane then processed with Maiia columns at flow of 1 ml/min using vacuum system. Once finished, 1 mL of "Washing buffer" (Maiia kit component) was added on the column of each sample at 1 ml / min flow rate then columns were centrifuged at 2000 g for 1 min each in a corresponding Eppendorf. After that, 35 µL of the desorption buffer SARCOSYL was added to each column and incubated at room temperature for 5 minutes and then centrifugated at 2,000 g for 1 min. The extract of each column was passed to an Amicon Ultra-0.5 30KDa filter and centrifuged at 14000 g for 20 min. After centrifugation, the filter placed inverted in an adapter Eppendorf previously

labeled with the sample code and centrifugated at 14000 g for 1 min. The supernatant of each column was aliquoted into 10 µl and stored at -20ºC till use.

2.5. Electrophoresis in gels from SAR-PAGE

The following operations were performed using electrophoresis equipment of the Invitrogen commercial house. A pre-made NuPAGE 10% bis-tris gel was used in electrophoresis. Electrophoresis was run at 125V, for a period of 3 hours under cooling conditions.

2.6. Immunodetection

The semi-dry transfer equipment was used according to the trans-blot semi-dry electrophoretic transfer cell instruction manual (BIO-RAD) .

The device was covered with the other electrode and then with the lid, and the system was connected to the power supply to start blotting according to the following conditions (1.54 mA / cm^2 constant, for 45 min).

2.7. Immunoblotting

After the transfer was finished, the immunoblotting process was started using automated BlotCycler according to BlotCycler Automated Western Blot Processor User Manual. Buffers and solutions for immunoblotting were prepared during the transfer process by preparing 4% and 0.8% milk solutions using PBS (phosphate buffered saline).

Primary antibody solution was prepared by placing 25ml of 0.8% milk in a falcon tube with 5ul of the primary antibody [5 ug / ml] (clone human anti-erythropoietin monoclonal antibody AE7A5, V00038BI) purchased from R&D, CLINILAB company.

Secondary antibody solution was prepared by placing 20 µl of 0.8% milk in an eppendorf tube with 2 µl of the secondary antibody labeled with HRP (Goat anti-mouse + HRP conjugate, E00072BI) purchased from EMD MILLIPORE company, then 2.5 µl of the previous solution were added to a falcon tube with 25 ml of 0.8% milk.

BlotCycler was prepared and fixed in a cold chamber at 4 °C and program conditions were reviewed to be (5 min Blocking (PBS) – 300 min 1st Antibody – 690 min 2nd Antibody – 5 min washing time) and washing cycles: 5 after blocking, 9 after 1st Antibody, 9 after 2nd Antibody.

The primary antibody solution was added in the corresponding receptacle P to the tray where the IMMOBILON 1 membrane was placed, and the secondary antibody solution was added in the corresponding S receptacle to the tray where the IMMOBILON 1 membrane was placed.

A volume of 3L of 1x PBS were added in the large container of the equipment and 25 ml of PBS 1x was added in the tray where the IMMOBILON 1 membrane was placed. Then, the process started.

At the end of the Blotcycler program, the IMMOBILON 1 membrane was placed in a new tray with 1x PBS under stirring at room temperature until it is read in the chemiluminescence chamber.

2.8. Chemiluminescence Detection

Chemiluminescence detection was performed according to the user manual of Amersham Image Quant 800 using the Amersham Image Quant 800 chemiluminescence chamber of General Electric Healthcare Life Sciences.

During the last minutes of washing with Blotcycler, the chemiluminescence reagent was prepared from the chemiluminescence detection kit purchased from THERMO SCIENTIFIC [Batch# T1270475] solution by mixing 1.5ml of each kit bottles (SuperSignal West Femto Luminol/ Enhancer solution + SuperSignal West Femto Peroxide Buffer).

The membrane was placed in a transparent tray containing the chemiluminescence reagent and incubated for 5 mins till totally soaked then placed in the chemiluminescence chamber to read at different exposure times (2 seconds – 10 seconds – automatic – 1 minute – 3 minutes).

3. Results

3.1. SAR-PAGE improved migration of erythropoietin

3.1.1. SAR-PAGE effect on non-PEGylated epoetins (r-EPO – NESP – EPO-FC)

The changes in the amount of SARCOSYL in sample and running buffers were accompanied by

an increase in the sensitivity of non-PEGylated (r-EPO – NESP – EPO-FC) recombinant erythropoietin due to the ability of SARCOSYL to only bind to the amino acid chain of the proteins thus leading to enhanced antibody binding and a sharper electrophoretic band.

The bands absolute volume is decreased by ten folds (10x) or more with the new amount of sarcosyl 0.12% w/v.

Group 1 (old method): large condensed shadowed bands and asymmetric band behavior was seen **(Figure 1A)**.

Group 2 (recent study): confirm, thin, sharp and symmetric identical bands behavior was seen compared to the old method **(Figure 2A).**

3.1.2. SAR-PAGE effect on PEGylated epoetins (MIRCERA)

The migration behavior of the PEGylated erythropoietin (MIRCERA) which is a PEGylated recombinant erythropoietin with a molecular weight of approximately 60 KDa was greatly enhanced using adjusted amounts of SARCOSYL in SAR-PAGE method in the sample and running buffers compared to the normal SAR-PAGE by enhancing the electrophoretic separation of the proteins through increased affinity of monoclonal anti-Epo antibody to the protein chain as sarcosyl does not bind to the polyethylene glycol (PEG)-chain of MIRCERA but only binding to the protein-part of it. Thus (PEG)-chain not migrated on SAR-PAGE and stayed at the place of application and did not produce the streaked bands (lanes) and stayed in their native uncharged state leading to a sharper electrophoretic separation with high sensitivity.

Group 1 (old method): large condensed shadowed bands and asymmetric non identical bands behavior was seen **(Figure 1B)**.

Group 2 (recent study): confirm, thin, sharp bands and symmetric identical bands behavior was seen compared to old method **(Figure 2B)**.

3.2. SAR-PAGE effect on immunological detection

Minor modification in the SAR-PAGE method by increasing the amount (concentration) of sarcosyl in the sample and running buffers affect the detection of ERYTHROPOIETIN by increasing the affinity to the monoclonal anti-EPO antibody by increasing the binding affinity only to the protein part of PEGYLATED proteins not to its PEG-Chain resulting in enhancing the electrophoretic characters of proteins which made it easier to migrate. Thus, lowering the absolute volume for each band by giving thin, confirm and sharp bands. Sarcosyl affects the immunological detection by increasing the affinity of the monoclonal anti-EPO antibody (Clone AE7A5) to the protien chain in both PEGylated and non-PEGylated epotiens thus leading to enhanced antibody binding and a sharper electrophoretic separation and detectable bands **(Figure 3)**.

3.3. SAR-PAGE effect on presence of mixed bands

Sarcosyl decreased the presence of mixed bands by enhancing the electrophoretic characters of the erythropoietins during migration by solubilizing only the protein part of both PEGylatd and non-PEGylated proteins which make it easier on epoetins to migrate through the gel by electrophoresis according to their molecular weights giving more detectable, separate and sharp bands **(Figure 4)**.

4. Discussion

Erythropoietin (EPO) is considered one of the most important tools in doping control world besides it is already found naturally in the human body and has been commercially available as recombinant human EPO (rEPO) for almost 20 years. Although the positive benefits of rEPO cannot be denied for those who suffer from anemia, athletes have also used the drug as an ergogenic aid **(Scott & Phillips, 2005; Vlad et al., 2018)**. It has replaced conventional “blood doping” as the drug of choice to improve performance in contests requiring aerobic potential. Testing for the drug in the past has proven difficult due to several factors. The similarities in structure and metabolism of EPO and rEPO allow those who choose to abuse rEPO to avoid detection. Low levels of EPO (around 10 mU/mL) are constantly secreted in sufficient quantities to compensate for normal red blood cell turnover. Common causes of cellular hypoxia resulting in elevated levels of EPO (up to 10 000 mU/mL) include any anemia, and hypoxemia due to chronic lung disease **(Mairbäurl, 2013)**.

Since erythropoietin plays an important role in the medical field treating anemia and kidney or renal failure patients **(Hayat et al., 2008)**, also plays an important role in doping control in athletes in the field of sport when it started to be detected in urine or blood samples in athletes who try to double the strength and power by exogenous erythropoietin

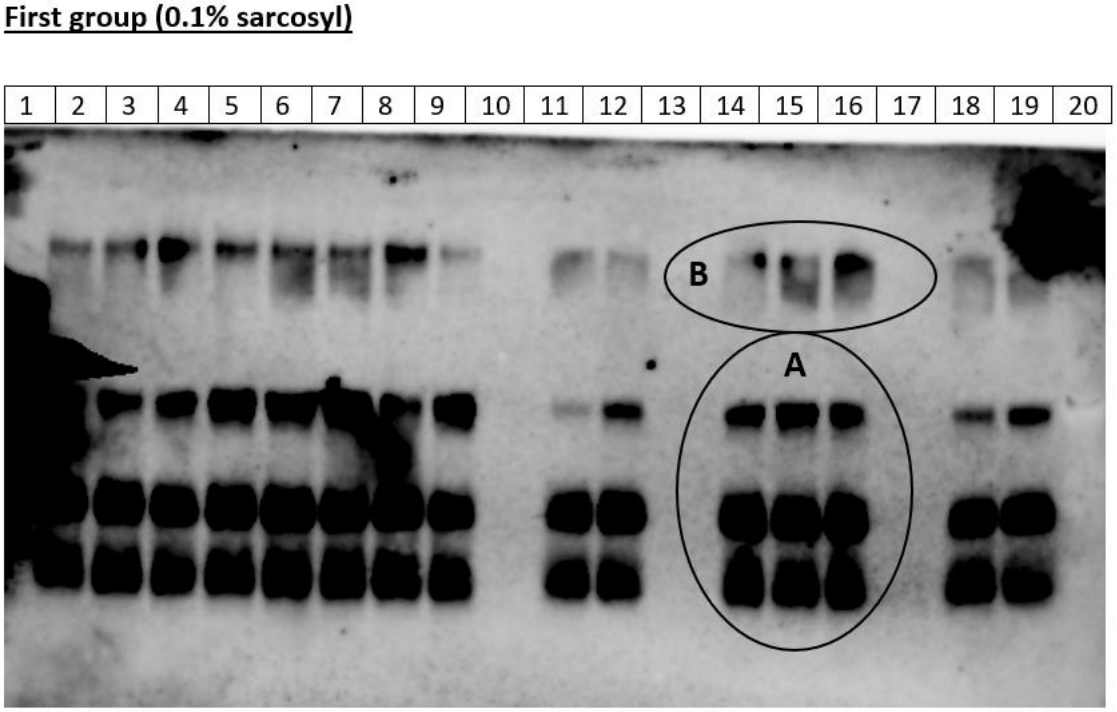

Figure 1. Detection of Non-PEGYLATED ERYTHROPOIETIN biosimilars **(A)** (EPO-FC, NESP & r-EPO) and PEGYLATED ERYTHROPOIETIN biosimilars **(B)** (MIRCERA) using 0.1% w/v SARCOSYL-PAGE with large condensed shadowed bands and asymmetric bands behavior.

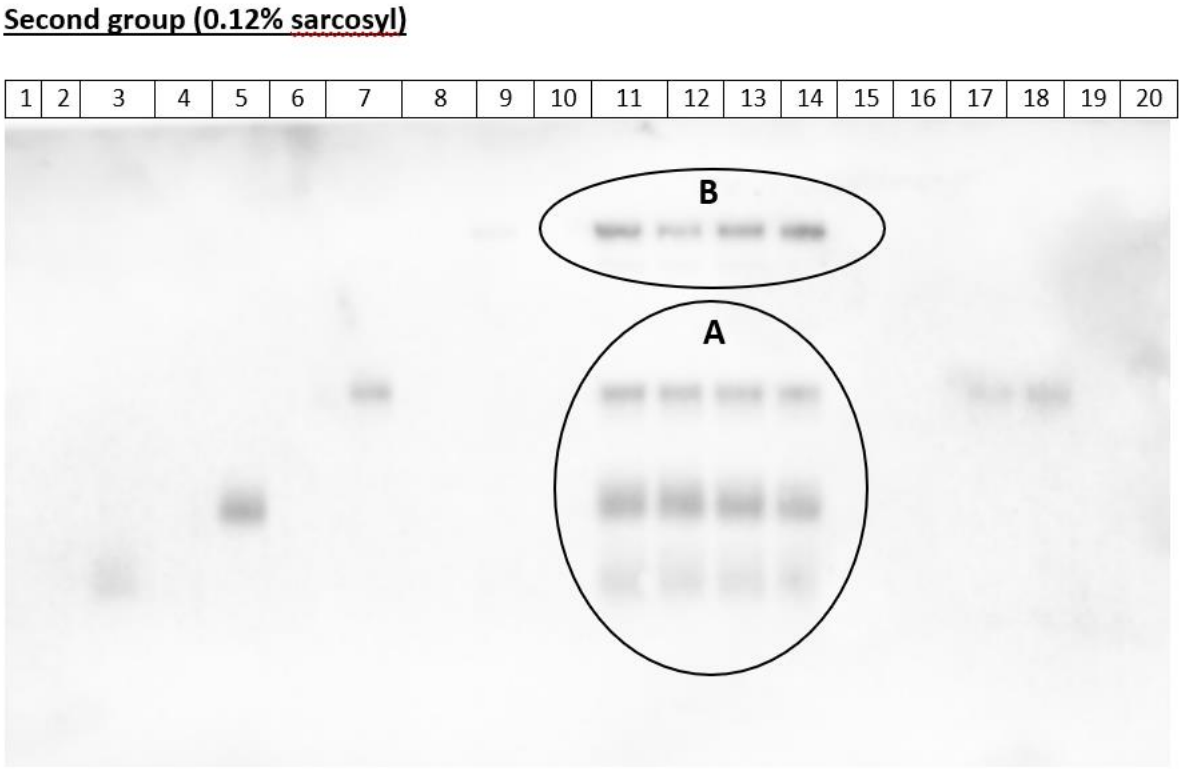

Figure 2. Detection of Non-PEGYLATED ERYTHROPOIETIN biosimilars **(A)** (EPO-FC, NESP & r-EPO) and PEGYLATED ERYTHROPOIETIN biosimilars **(B)** (MIRCERA) using 0.12% w/v SARCOSYL-PAGE with confirm, thin, sharp bands and symmetric identical bands behavior.

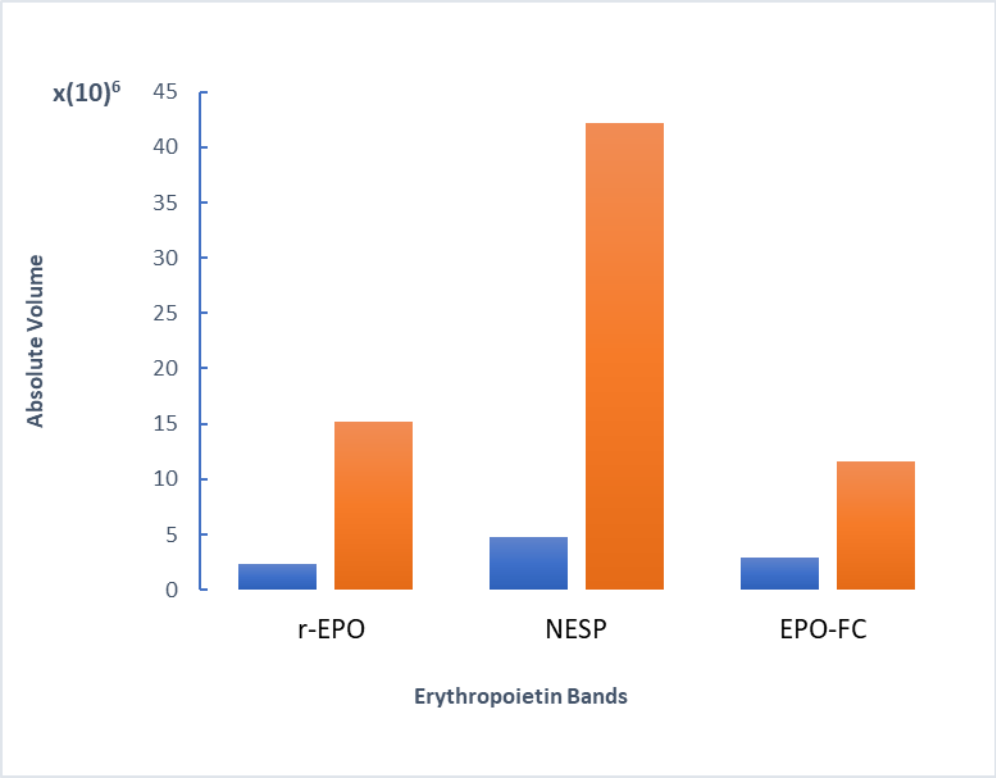

Figure 3. Comparison between 0.1% and 0.12% SARCOSYL-PAGE western blotting bands results by blotting the absolute volume for each band of the non-PEGYLATED Erythropoietin biosimilars, 0.1% SARCOSYL bands absolute volume is much larger than 0.12% SARCOSYL bands by 10 folds or more.

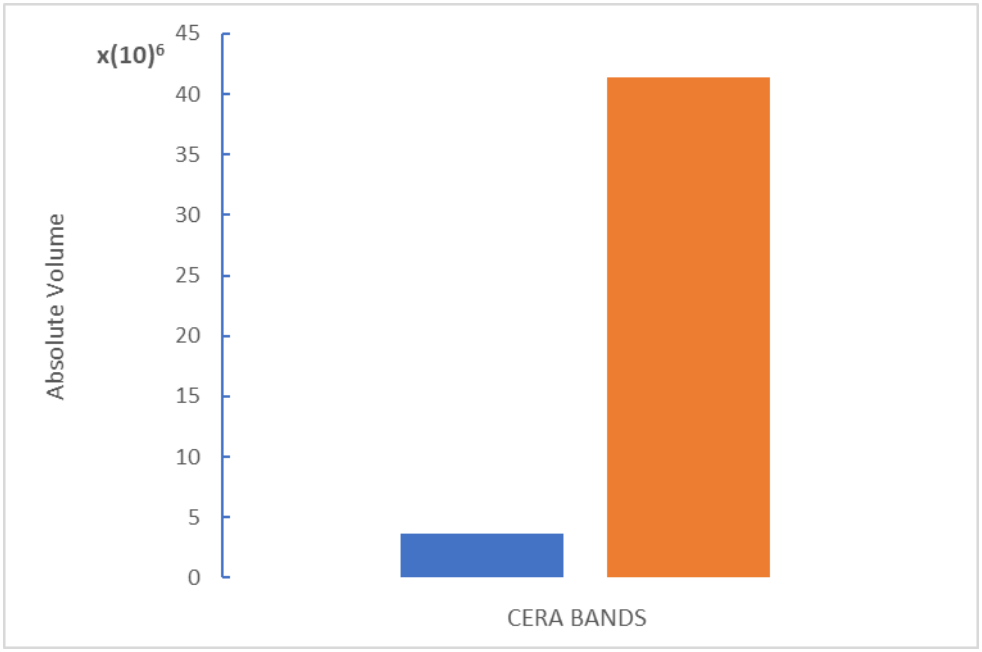

Figure 4. Comparison between 0.1% and 0.12% SARCOSYL-PAGE western blotting bands results by blotting the absolute volume for each band of the PEGYLATED Erythropoietin biosimilars, 0.1% SARCOSYL bands absolute volume is much larger than 0.12% SARCOSYL bands by 10 folds or more.

doses **(Schoener & Borger, 2024)**.

Here was the need for a new method or technique to be applied on same conditions that may face the doping agencies to help detection of erythropoietin in athlete's samples for a fair play sport **(Heuberger et al., 2019)**.

Studies show that detection of erythropoietin using SDS-PAGE gives reasonable results with all epoetin's except for MIRCERA **(Martin et al., 2022a; Yasuoka et al., 2023)** which is a PEGylated recombinant erythropoietin frequently used for the treatment of anemia although if can detect all of the non-PEGylated forms of erythropoietin **(Reichel et al., 2019)**.

Detection of recombinant erythropoietin biosimilars (PEGylated and non-PEGylated) forms by isoelectric focusing is relies on separation by molecular weight during migration of proteins when an electric charge applied **(Stutz, 2023)**.

The current study showed that the use of SAR-PAGE in detection of erythropoietin gives more sensitivity for the majority of epoetins than SDS-PAGE specially in case of MIRCERA (PEGylated epoetin beta) **(Cavalcanti et al., 2019; Reihlen et al., 2021)** which its sensitivity is drastically reduced with SDS-PAGE. Redesigning SDS-PAGE by exchanging the SDS for sarcosyl in the sample and running buffers specifically enhanced the sensitivity for MIRCERA **(Reichel et al., 2019; Martin et al., 2022b)**.

In this study, changes in the amount of sarcosyl in sample and running buffers were accompanied by an increase in the sensitivity of PEGylated (r-EPO – NESP – EPO-FC) and non-PEGylated (MIRCERA) recombinant erythropoietin due to the ability of sarcosyl to only bind to the amino acid chain of the PEGylated proteins thus leading to enhanced antibody binding and a sharper electrophoretic band **(Reichel, 2012; Reihlen et al., 2021)**.

The migration behavior of the PEGylated erythropoietin (MIRCERA) was greatly enhanced using adjusted amounts of sarcosyl 0.12% W/V in SAR-PAGE method in the sample and running buffers compared to the normal SAR-PAGE **(Reichel et al., 2019)**. by enhancing the electrophoretic separation of the proteins through increased affinity of monoclonal anti-Epo antibody to the protein chain **(Heiland et al., 2019)** as sarcosyl only binding to the protein-part of MIRCERA but not to its polyethylene glycol (PEG)-chain which not migrated on SAR-PAGE and stayed at the place of application and did not produce the streaked bands (lanes) and stayed in their native uncharged state leading to a sharper electrophoretic separation with high sensitivity **(Lasne et al., 2009; Yasuoka et al., 2023)**.

Previous studies show that the altered Western blotting behavior of MIRCERA was due to its PEGylation. Hence, PEGs of different average molecular masses were separated on SDS-PAGE **(Zheng et al., 2007)**. It was demonstrated that SDS binds to PEG and thus leads to the migration of the uncharged PEG molecules in the electric field. However, due to the limited solubilizing power of SDS, PEGs – regardless of molecular size – migrate as broad and smeared bands on SDS-PAGE **(Reihlen et al., 2021)**.

As evidenced so far, the binding of SDS to both the protein and PEG parts of MIRCERA led to the broad band with the atypical (for an epoetin beta) but significant (for PEG) density distribution. And as further substantiated by the smeared bands, SDS **(Reichel et al., 2009b; Nowakowski et al., 2014)** – as an anionic detergent – was not capable of fully solubilizing PEGs regardless of their molecular size. Hence, it was concluded that the decreased sensitivity of MIRCERA on Western blots might be due to an SDS-based solubility problem of MIRCERA's PEG-part or an SDS-based problem concerning a non-homogenous binding of SDS to the polymer **(Bhalani et al., 2023)**.

Consequently, two strategies seemed to be promising for solving the problem: either (1) using a detergent with higher solubilizing power for PEGs than SDS (or using SDS at an increased concentration), or – less obvious – (2) using or locating a detergent that does only bind to the protein-part of MIRCERA **(Reichel et al., 2009a)** but not to the PEG-moiety. Consequently, it was decided to replace the SDS in SDS-PAGE by anionic detergents with different physicochemical properties than SDS but without changing the electrophoretic system **(Bjørnestad & Lund, 2023)**.

According to a previous study in order to identify the reason for the excellent performance characteristics of SAR-PAGE for MIRCERA, PEGs of different molecular sizes (from 1500 to 35000 Da) were applied on gels and subsequently PEG stained **(Reichel et al., 2009a)**. Amazingly, PEGs did not migrate on SAR-PAGE; hence they stayed at the place of application and did not

produce the streaked bands (lanes) as observed on SDS-PAGE. SARCOSYL was obviously not binding to polyethylene glycol and was thus leaving PEGs in their native uncharged state **(Reihlen et al., 2021)**.

Consequently, and due to the fact that sarcosyl was only binding to the protein part of MIRCERA, the obtained band sharpness of MIRCERA on SAR-PAGE could be explained. The strong solubilizing characteristics of SDS **(Reichel et al., 2009b; Reichel et al., 2019)** for PEGs were the reason for the poor resolution of MIRCERA on SDS-PAGE **(Zheng et al., 2007; Heiland et al., 2019)**, thus leading to the fact that SAR-PAGE is practically proven its ability to detect erythropoietin much better than SDS-PAGE especially for the PEGylated epoetin's (MIRCERA).

In this study, the minor modification in the SAR-PAGE method was done by increasing the amount (concentration) of sarcosyl in the sample and running buffers affecting the detection of erythropoietin by increasing the affinity to the monoclonal anti-EPO antibody **(Lasne et al., 2009; Reichel et al., 2019)** via increasing the binding affinity only to the protein part of PEGYLATED proteins not to its PEG-Chain resulting in enhancing the electrophoretic characters of proteins which make it easier to migrate. Thus, lowering the absolute volume for each band giving thin, confirm and sharp bands **(Reichel, 2012)**.

However, the increase in band sharpness on SAR-PAGE also increased the antibody binding efficiency. On SDS-PAGE **(Martin et al., 2022b)** the monoclonal antibody was shown to be bound only to the part of the band that contained the lowest amount of MIRCERA, but no binding occurred to the region of the highest MIRCERA concentration. As demonstrated by isoelectric focusing of MIRCERA, PEGyaltion did not significantly decrease the affinity of the antibody to its N-terminal epitope, i.e. the sensitivity for MIRCERA on IEF-PAGE was comparable to the sensitivity for non- PEGylated epoetins **(Lasne et al., 2009; Fisusi et al., 2020)**. Hence, it is hypothesized that the formation of micelles containing the detergent-solubilized PEG-part **(Bjørnestad & Lund, 2023)** as well as the detergent-solubilized protein-part hinder the accessibility of the N-terminal epitope for the antibody (clone AE7A5; directed against a linear epitope consisting of the first 26 amino acids of the erythropoietin N-terminus) **(Voss et al., 2021)**. If anionic detergent-based micelles are formed that consist of only the solubilized protein-part of MIRCERA then no interference occurs **(Bjørnestad & Lund, 2023)**. The resolution of the band was not disturbed, and antigen-antibody interactions were not hindered. Consequently, the less anionic detergent is able to interact with the PEG-group of the protein (as is the case for sarcosyl) the more unaltered the binding behavior of the monoclonal antibody becomes and the more sensitively the MIRCERA can be detected.

Sodium N-lauroyl sarosinate (SARCOSYL) is an amino acid-based detergent (N-dodecanoyl-N-methylglycine sodium salt, an acyl sarcosinate), which has been typically used in molecular biology (for example, in guanidine-containing RNA purification buffers) and protein chemistry for solubilizing membrane proteins **(Arakawa et al., 2024)**.

Acyl sarcosinates have been described as ‘interrupted soaps. They contain a carboxylate (head) group and an additional amide function, which greatly enhances the surfactant properties (a structural element also found in GDOC) **(Hibbs, 2006; Reichel, 2012)**. The molecular weight (MW) and CMC of N-lauroyl sarosinate are similar to SDS (SAR: MW 293.4, CMC 14.6 mM; SDS: MW 288.4, CMC 7– 10 mM; supplier information), while the aggregation number is much lower (2 instead of 62) and comparable to the aggregation number of bile salt-related detergents. SARCOSYL-PAGE performed excellent on Western blots by resolving recombinant EPO especially MIRCERA in a sharp band and simultaneously keeping the resolution of rhEPOs and uhEPO on the performance level of SDS-PAGE **(Reichel et al., 2019)**.

5. Conclusion

Current data proved that the increase in sarcosyl concentration to 0.12 % w/v in the detection of human recombinant erythropoietin and its biosimilars using gel electrophoresis by SAR-PAGE, helps in the improvement of EPO detection to get more specific and sharp detectable bands through the following mechanisms:

1. Increasing the affinity to anti-human EPO.
2. Decreasing the solubilizing characters for PEGylated part of EPO.
3. Increasing affinity of binding to only the protein part of EPO.
4. Facilitates the migration process of EPO biosimilars during electrophoresis.

References

Arakawa, T., Niikura, T., Kita, Y., & Akuta, T. (2024). Sodium Dodecyl Sulfate Analogs as a Potential Molecular Biology Reagent. Curr Issues Mol Biol, 46(1), 621-633.

Bhalani, D., Kakkad, H., Modh, J., Ray, D., Aswal, V. K., & Pillai, S. A. (2023). Molecular insights into the aggregation and solubilizing behavior of biocompatible amphiphiles Gelucire® 48/16 and Tetronics® 1304 in aqueous media. RSC Adv, 13(41), 28590-28601.

Bjørnestad, V. A., & Lund, R. (2023). Pathways of Membrane Solubilization: A Structural Study of Model Lipid Vesicles Exposed to Classical Detergents. Langmuir, 39(11), 3914-3933.

Cavalcanti, R. T. C., Teixeira, P. A. C., Levy, R. S., Pereira, H. M. G., & Neto, F. R. A. (2019). Detection of ESAs in equine urine and blood by SAR-PAGE. Drug Test Anal, 11(6), 772-781.

Desharnais, P., Naud, J. F., & Ayotte, C. (2013). Desialylation improves the detection of recombinant erythropoietins in urine samples analyzed by SDS-PAGE. Drug Test Anal, 5(11-12), 870-876.

Fisusi, F., Brandy, N., Wu, J., & Akala, E. O. (2020). Studies on polyethylene glycol-monoclonal antibody conjugates for fabrication of nanoparticles for biomedical applications. J Nanosci Nanomed, 4(2), 1-9.

Hayat, A., Haria, D., & Salifu, M. O. (2008). Erythropoietin stimulating agents in the management of anemia of chronic kidney disease. Patient Prefer Adherence, 2, 195-200.

Heiland, C. E., Masquelier, M., Bhuiyan, H., & Ericsson, M. (2019). A simple method to immunopurify erthyropoiesis stimulating agents from urine, aiming to optimize erythropoietin screening by SAR-PAGE. Drug Test Anal, 11(11-12), 1666-1674.

Heuberger, J., van Eenoo, P., Rotmans, J. I., Gal, P., Stuurman, F. E., Post, T. E., Daniels, J. M. A., Ram, H., de Hon, O., Burggraaf, J., Cohen, A. F. (2019). Sensitivity and specificity of detection methods for erythropoietin doping in cyclists. Drug Test Anal, 11(9), 1290-1301.

Hibbs, J. (2006). Anionic Surfactants. In Chemistry and Technology of Surfactants (pp. 91-132).

Jelkmann, W. (2013). Physiology and pharmacology of erythropoietin. Transfus Med Hemother, 40(5), 302-309

Kielkopf, C. L., Bauer, W., & Urbatsch, I. L. (2021). Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of Proteins. Cold Spring Harb Protoc, 2021(12), 10.1101/pdb.prot102228.

Lasne, F., Martin, L., Martin, J. A., & de Ceaurriz, J. (2009). Detection of continuous erythropoietin receptor activator in blood and urine in anti-doping control. Haematologica, 94(6), 888-890.

Mairbäurl, H. (2013). Red blood cells in sports: effects of exercise and training on oxygen supply by red blood cells. Front Physiol, 4, 332.

Martin, L., Martin, J. A., Audran, M., & Marchand, A. (2022a). An optimized SDS-PAGE protocol with a new blotting system for the initial testing procedure of ESAs in doping control. Drug Test Anal, 14(1), 181-187.

Martin, L., Martin, J. A., Audran, M., & Marchand, A. (2022b). An optimized SDS-PAGE protocol with a new blotting system for the initial testing procedure of ESAs in doping control. Drug Test Anal, 14(1), 181-187.

Nowakowski, A. B., Wobig, W. J., & Petering, D. H. (2014). Native SDS-PAGE: high resolution electrophoretic separation of proteins with retention of native properties including bound metal ions. Metallomics, 6(5), 1068-1078.

Reichel, C. (2012). SARCOSYL-PAGE: a new electrophoretic method for the separation and immunological detection of PEGylated proteins. Methods Mol Biol, 869, 65-79.

Reichel, C., Abzieher, F., & Geisendorfer, T. (2009a). SARCOSYL-PAGE: a new method for the detection of MIRCERA- and EPO-doping in blood. Drug Test Anal, 1(11-12), 494-504.

Reichel, C., Gmeiner, G., Reihlen, P., Thevis, M., & Schanzer, W. (2019). SARCOSYL-PAGE: Optimized Protocols for the Separation and Immunological Detection of PEGylated Proteins. In B. T. Kurien & R. H. Scofield (Eds.), ELECTROPHORETIC SEPARATION OF PROTEINS: Methods and Protocols (Vol. 1855, pp. 131-149). Totowa: Humana Press Inc.

Reichel, C., Kulovics, R., Jordan, V., Watzinger, M., & Geisendorfer, T. (2009b). SDS-PAGE of recombinant and endogenous erythropoietins: benefits and limitations of the method for application in doping control. Drug Test Anal, 1(1),

43-50.

Reihlen, P., Blobel, M., Weiß, P., Harth, J., Wittmann, J., Leenders, F., & Thevis, M. (2021). Introduction of a PEGylated EPO conjugate as internal standard for EPO analysis in doping controls. Drug Test Anal, 16(8), 743-749.

Reihlen, P., Volker-Schanzer, E., Majer, B., & Schanzer, W. (2012). Easy-to-use IEF compatible immunoaffinity purification of Erythropoietin from urine retentates. Drug Testing and Analysis, 4(11), 813-817.

Schoener, B., & Borger, J. (2024). Erythropoietin Stimulating Agents. In StatPearls. Treasure Island (FL): StatPearls Publishing LLC.

Scott, J., & Phillips, G. C. (2005). Erythropoietin in Sports: A New Look at An Old Problem. Curr Sports Med Rep, 4(4), 224-226.

Stutz, H. (2023). Advances and applications of capillary electromigration methods in the analysis of therapeutic and diagnostic recombinant proteins – A Review. J Pharm Biomed Anal, 222, 115089.

Vlad, R. A., Hancu, G., Popescu, G. C., & Lungu, I. A. (2018). Doping in Sports, a Never-Ending Story? Adv Pharm Bull, 8(4), 529-534.

Voss, S. C., Orie, N. N., El-Saftawy, W., Saghbazarian, S., Al-Kaabi, A., Georgakopoulos, C., Athanasiadou, I., Mohamed-Ali, V., Al-Maadheed, M. (2021). Horseradish-peroxidase-conjugated anti-erythropoietin antibodies for direct recombinant human erythropoietin detection: Proof of concept. Drug Test Anal, 13(3), 529-538.

Yasuoka, Y., Izumi, Y., Sands, J. M., Kawahara, K., & Nonoguchi, H. (2023). Progress in the Detection of Erythropoietin in Blood, Urine, and Tissue. Molecules, 28(11), 4446.

Zheng, C. Y., Ma, G., & Su, Z. (2007). Native PAGE eliminates the problem of PEG–SDS interaction in SDS-PAGE and provides an alternative to HPLC in characterization of protein PEGylation. Electrophoresis, 28(16), 2801-2807.

Zivot, A., Lipton, J. M., Narla, A., & Blanc, L. (2018). Erythropoiesis: insights into pathophysiology and treatments in 2017. Mol Med, 24(1), 11.